Synopsis

The glial cells of the brain and the peripheral nervous system retain the capacity to divide and proliferate throughout the lifespan of an individual and thereby have the propensity to give rise to the most adult neurological tumours. Among them, the tumours which arise from different kinds of glial cells are referred to as gliomas. Of the various types of gliomas, astrocytomas are the most common central nervous system neoplasms which make upto 60% of all the primary brain tumours. Being the most prevalent type, the WHO classifies them into grades ranging from I to IV based on their intensity of malignancy. Grade IV astrocytoma or Glioblastoma (GBM) is considered to be the most malignant form with a median survival of 14.6 months, in spite of all therapeutic modalities. GBM is further classified as primary and secondary GBM. Primary GBM manifests *de novo* without any early history of pre-malignant lesions, on the other hand secondary GBM arises progressively from lower grades over a period of 5-10 years.

Like other malignancies, GBM also arises from various genetic and epigenetic variations. Epigenetic variations include all such mitotically and meiotically heritable traits that do not involve changes in DNA sequence. There are three major areas of epigenetics - DNA methylation, histone modifications and non-coding RNAs which are known to have profound effects on gene expression. A lot being known about the genetic derailments in GBM, in this study we looked into the epigenetic aspects of GBM. In our lab, we have carried out various high throughput studies, which unveiled the distorted landscape of DNA methylation and miRNA expression in GBM. This indicates that, in addition to the genetic mechanisms of gene alterations like mutations, copy number aberrations, protein coding genes are also affected by changes in methylation as well as by miRNA misregulation. The study has been divided into two parts. Part one of the study deals with the identification of *chromobox homolog 7 (Cbx7)*, as a hypermethylated and downregulated gene in GBM. More importantly, Cbx7 is a member of the polycomb repressive complex and brings about its function through chromatin modifications. Here we have investigated the role of Cbx7 in gliomagenesis, and why it has to be silenced by methylation for tumorigenesis to ensue. In part two, we elucidated two unique ways of miRNA regulation in GBM. In the first section, we identified miR-326 as a PI3 kinase regulated miRNA and demonstrated its tumour suppressive role in GBM. In the other section, we analysed the copy number aberration data from TCGA and identified miR-

4484 as a miRNA subjected to deletion in GBM. We further went ahead to demonstrate its growth suppressive role in GBM.

Part 1: Epigenetic regulation of the chromatin modifier Cbx7; chromobox homolog 7

DNA methylation is involved in the normal cellular control of expression and thereby plays a crucial role in maintaining the homeostasis of the cell. The phenomenon of DNA methylation keeps the various loci of the genome such as the germline specific genes and the repetitive transposable elements silenced, whereas the tumour suppressors and other growth modulator genes are spared from the methylation induced gene repression. One of the important steps that promote tumorigenesis is aberrant hypermethylation, which leads to the silencing of tumour-suppressor genes. Another important epigenetic phenomenon that affects the transcriptibility of the genome is histone modifications, which control the accessibility of the chromatin to the transcriptional machinery. In this section, we identified Cbx7, which happens to be an essential component of the chromatin modifying machinery, as an epigenetically regulated gene in GBM. We observed from the methylation array carried out in our lab, that Cbx7 was one of the highly methylated genes. We also validated that Cbx7 is downregulated in GBM and the same observation was further corroborated from other data sets. The hypermethylated state of Cbx7 was confirmed by DNA bisulphite sequencing and the expression levels of Cbx7 also got alleviated after 5-Aza-2′-deoxycytidine treatment, which is a DNA methylation inhibitor. This indicated that the down regulation of Cbx7 could be attributed to the methylation of its promoter region. In order to figure out the role of Cbx7 in GBM, we carried out transcriptome analysis of Cbx7 overexpressing cells compared to vector control condition by RNA sequencing. Gene ontology analysis revealed a significant enrichment of pathways involved in cell cycle, migration and invasion like processes. In fact, the exogenous overexpression of Cbx7 leads to cell death, reduced colony formation, retarded migration and invasion of cells. In order to explain the above phenotypes brought about by the exogenous expression of Cbx7, we further examined the RNA sequencing data and observed that many of the top most downregulated genes in Cbx7 overexpression state belonged to the Hippo signaling pathway. The effectors of the Hippo pathway, YAP and TAZ which essentially antagonize the pathway activity, are well known for their role in proliferation, migration and invasion in cancer. So we carried out a Gene Set Enrichment

Analysis (GSEA) and found that there was a significant negative enrichment of YAP/TAZ targets in the Cbx7 regulated gene set. We validated some of these targets that were downregulated by Cbx7 overexpression. One of the most downregulated genes that we validated was *Connective Tissue Growth Factor (CTGF),* which also happens to be a bonafide target of YAP/TAZ. Independent downregulation of CTGF also resulted in reduced migration, thereby phenocopying the effects as were produced by Cbx7 overexpression. Moreover, we also observed that SAPK/JNK was the only kinase whose activity was abolished upon Cbx7 overexpression. Since CTGF is known to activate SAPK/JNK, we assessed the SAPK/JNK activity upon CTGF silencing. We found that levels of phospho-SAPK/JNK were significantly reduced in CTGF silenced condition. In addition to that, the inhibition of the SAPK/JNK by synthetic inhibitor also hampered the migration ability of the cells. We were also able to rescue the loss of migratory potential of glioma cells by the exogenous overexpression of CTGF in Cbx7 stable background. A similar rescue was also achieved by the overexpression of a constitutively active form of SAPK/JNK. This indicates that Cbx7 activates Hippo pathway to inhibit YAP/TAZ dependent transcription, resulting in the downregulation of CTGF, thereby inhibiting CTGF mediated activation of SAPK and thus resulting in the inhibition of glioma cell migration.

PART 2: ROLE OF MIRNAS IN GLIOMA DEVELOPMENT AND PROGRESSION

miRNAs are a class of small non-coding RNAs that are not translated into functional proteins but still contribute to numerous cellular processes, thereby adding yet another realm of regulation and control. miRNAs bring about gene regulation at the post-transcriptional level, either by degrading the mRNA or by translational repression and in this manner fine tune the expression of protein coding genes. miRNAs are often located in the most fragile sites of the genome which exposes them to grave genetic alterations, thus providing a circumstantial evidence of their etiological role in tumorigenesis. In a malignant state, miRNAs have been found to play pivotal roles in cellular transformation by altering various cellular phenotypes. Owing to their participation in diverse cellular functions, miRNAs have gained a strong foothold in gene regulation. Though a lot has been deciphered about the functional aspect of miRNAs, not much is known about the precise mechanisms which lead to their misregulation and therefore demands in-depth

study. The expression of miRNAs can be modulated by a variety of genetic and epigenetic mechanisms.

Section I: Role of miR-326 – a PI3 kinase regulated miRNA, in gliomagenesis

The TCGA group in the year 2008 identified three major pathways which go disarray in GBM. These include the pro-tumorigenic receptor tyrosine kinase (RTK) pathway, and the p53 and the pRB tumour-suppressive pathways. The RTK signalling includes the PI3 kinase pathway, which is pivotal in gliomagenesis and many other cancers. This directed us to elucidate the set of miRNAs which are controlled by the aberrant functioning of the PI3 kinase pathway. We used synthetic inhibitor LY294002 to abrogate the PI3 kinase signalling and examined the miRNA profile in two glioma cell lines U87 and U251, which have an activated PI3 kinase pathway. Indeed the abrogation of the PI3 kinase pathway resulted in the modulation of a wide array of miRNAs. We validated miR-326 as one of the miRNAs that was upregulated upon PI3 kinase pathway abrogation. Furthermore, we observed that miR-326 was a down regulated miRNA in GBM and different glioma cell lines, as well as in many other publicly available data sets. We also observed that miR-326 is an intragenic miRNA and its host gene *Arrestin β1 (ARRB1)* also exhibited similar upregulation upon PI3K pathway inhibition. Over-expression of miR-326 resulted in various anti-tumorigenic affects like reduced proliferation, reduced migration and colony suppression. In order to find the targets of miR-326, we analysed the transcriptome by RNA sequencing upon pre-miR-326 transfection. We shortlisted and validated some of the genes which were getting regulated through miRNA over-expression and also explain the functional role of miR-326.

Section II: Role of miR-4484 – a copy number deleted miRNA, in gliomagenesis

In the TCGA study mentioned above, it was also unfurled that there are many genes in the RTK, p53 and pRB signalling pathways which are made dysfunctional through gene deletions and amplifications. We envisaged whether it is only the protein coding genes which are subjected to such regulations or the non-coding genes like miRNAs as well. In this pursuit, we identified miR-4484 as one of the miRNAs located in the deleted region of *uroporphyrinogen III synthase (UROS)* gene in the chromosome 11 of the GBM genome. As conceived, miR-4484 was observed to be a downregulated miRNA in association with its host gene UROS. We further elucidated that the downregulation was

due to the co-deletion of a locus harbouring both the protein coding gene and the miRNA. In addition, upon over-expression of miR-4484, we observed reduced migration and colony formation, indicating its role as a tumour–suppressor. For seeking the targets of miR-4484, we extracted RNA from miR-4484 over-expression condition and subjected it to RNA sequencing. We shortlisted and validated some of the genes which were getting regulated through miRNA over-expression and possibly explain the functional role of miR-4484.